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HLA Monomers as a Tool to Monitor Indirect Allorecognition

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Background. Recognition of donor antigens can occur through two separate pathways: the direct pathway (non-self HLA on donor cells) and the indirect pathway (self-restricted presentation of donor derived peptides on recipient cells). Indirect allorecognition is important in the development of humoral rejection; therefore, there is an increasing interest in the monitoring of indirect alloreactive T-cells. We have used an in vitro model to determine the optimal requirements for indirect presentation and assessed the risk for semidirect presentation in this system.

Methods. HLA-typed monocyte-derived dendritic cells (moDCs) were incubated with cellular fragments or necrotic cells and incubated with either indirect or direct alloreactive T-cell clones. T-cell reactivity was measured through proliferation or cytokine secretion. HLA-typed moDC, monocytes, or PBMCs were incubated with HLA class I monomers, in combination with either direct/indirect T-cell clones.

Results. Although both were efficiently taken up, alloreactivity was limited to the semi-direct pathway, as measured by allospecific CD4 (indirect) and CD8 T-cell clones (direct) when cells were used. In contrast, HLA-A2 monomers were not only efficiently taken up but also processed and presented by HLA-typed moDC, monocytes, and PBMCs. Activation was shown by a dose-dependent induction of IFN-γ production and proliferation by the CD4 T-cell clone. Antigen presentation was most efficient when the monomers were cultured for longer periods (24–48 hr) in the presence of the T-cells. Using this method, no reactivity was observed by the CD8 T-cell clone, confirming no semidirect alloreactivity.

Conclusion. We have developed a system that could be used to monitor indirect alloreactive T-cells.

Keywords: Indirect allorecognition, Immune monitoring, HLA, Allopeptide.

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Transplantation has become the standard therapy in end-stage renal failure, leading to an increase in quality of life and survival (*1*). Despite immunosuppressive regimes that are very effective in reducing acute rejection, chronic rejection remains a major cause of allograft loss (*2*).

T-cells are a major driving force in mediating allograft rejection (*3*). Recipient CD4+ T-cells can recognize the allograft either through recognition of donor HLA-peptide complexes on donor antigen-presenting cells (APC, direct), or by recognizing self-restricted donor HLA-derived peptides on recipient APCs (indirect). A third pathway (semi-direct) has been postulated in which recipient T-cells recognize intact donor HLA-peptide complexes on recipient APCs (*4, 5*).

T-cells with direct alloreactivity have been shown to play a dominant role in acute rejection, primarily in the early phase after transplantation, when APCs from donor origin are still available (*6–8*). T-cells with indirect alloreactivity are correlated with chronic rejection in humans (*7, 9, 10*). The importance of indirect allorecognition in allograft rejection is multiple and was recently reviewed (*11*). Briefly, it can provide help

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and thus induce alloreactive cytotoxic CD8 T-cells (*12, 13*). Perhaps more importantly, indirect alloreactive CD4 T-cells are the only cells that can provide help to alloreactive B-cells as shown in animal models (*13–15*). These cells have also been implicated in the regulation of tolerance (*16*). Regulatory T-cells (Treg) from transplant recipients were shown to have indirect alloreactivity (*17, 18*). Furthermore, they were capable of suppressing both the direct and indirect pathways of allorecognition (*19*). Animal studies have shown that Treg with indirect allospecificity can abrogate alloantibody formation and mediate transplant tolerance (*20–22*). Recently, natural Treg were shown to inhibit direct but not indirect allorecognition (*23*). Although both pathways have been known for decades, assays monitoring indirect T-cell alloreactivity have not become routine, and as of yet, no clinical assay is available (*11*).

Methods to monitor direct alloreactivity include the mixed leukocyte reaction (MLR), the cytotoxic T-cell precursor assay and more recently, the IFN-γ enzyme linked immunosorbent spot (ELISPOT). The assays give a good indication of the potency of directly reactive T-cells (*24–27*). Methods to measure indirectly reactive T-cells have been previously reviewed and offer many complications (*28*). These include the use of donor cell fragments as a source of donor antigens, which are presented in the context of self-HLA class II. In theory, this method allows the full repertoire of alloantigen available. In practice, this assay is difficult and not very reproducible. Furthermore, reactivity may be due to the semi-direct pathway (*29, 30*). Synthetic peptides, which correspond with the mismatched donor antigens have also been used (*31–33*). This method has a higher reproducibility but reactivity may be directed at neo-epitopes that are not available in vivo. The third method makes use of the trans vivo delayed-type hypersensitivity (DTH) model, in which recipient cells and donor cellular fragments are injected into the footpad of naive mice (*34–36*). Donor cell alloreactivity leads to a DTH response that can be measured by the footpad swelling. The advantage is that a global alloresponse can be measured (*37*). However, this approach is very complex and technically too difficult to be used as a diagnostic assay.

The aim of this study was to develop a reliable and easy tool to monitor indirect allorecognition in renal transplant patients that could be used in a clinical setting.

RESULTS

Evidence for Semidirect Rather Than Indirect Allorecognition When Donor Cell Fragments Are Used as Exogenous Antigen Source

To set up and validate a model for indirect allopresentation, a previously characterized CD4 T-cell clone (referred to as 4.44) recognizing HLA-A2 peptide restricted by HLA-DR1 was used (*38*). Incubation of 4.44 with its native ligand in the form of Epstein-Barr virus (EBV) transformed B-cell line (EBV-LCL) expressing HLA-DR1 and HLA-A2 led to IFN-γ secretion (Fig. 1A). Clone 4.44 did not react with HLA-DR1+/HLA-A2- EBV-LCLs, but IFN-γ secretion could be induced when cells were pulsed with HLA-A2 peptides containing the relevant epitope (Fig. 1B).

A CD8 T-cell clone (referred to as 1E2) that recognizes HLA-A2 (*39*) was used as a readout to exclude semi-direct alloreactivity. Both T cell clones were cultured with single antigen lines (SALs) expressing only HLA-A2 (SAL-A2). T-cell reactivity as measured by IFN-γ was limited to the 1E2 T-cells, confirming specificity of both clones (Fig. 1C).

To investigate the process of indirect alloreactivity, monocyte-derived dendritic cells (moDC) from HLA-typed donors (HLA-DR1+/HLA-A2-) were used as APCs and were loaded with peptides or fragmented SAL-A2 cells as antigen source. After 48 hr, clone 4.44 was added, but apart from the positive control, no indirect alloreactivity could be measured in any of the conditions (Fig. 1D). In contrast, under similar conditions of loading fragments, when clone 1E2 was used, low but significant production of IFN-γ could be detected in three of five experiments (Fig. 1E). This indicates that the use of cell fragments might result in semidirect allorecognition when cocultured with moDC. No reactivity was observed when fragments and 1E2 were cultured alone. 1E2 activation was confirmed in all conditions where viable SAL-A2 were used.

Processing of Necrotic Cells Leads to Semidirect Allorecognition

Professional APCs have specialized mechanisms for the uptake of apoptotic or necrotic cells. Therefore, we investigated whether dead cells could be a more efficient way of loading alloantigen, without the risk of semidirect allorecognition. Viable or necrotic SAL-A2 cells were used as previously described (*40*). HLA-A2 expression on SAL-A2 was confirmed on viable and necrotic (Nec) SAL-A2 (Fig. 2A). Moreover, SAL-A2 were stimulated with IFN-γ to increase HLA-A2 expression on these cells (Fig. 2B). Uptake of dead cells, but not viable cells, was confirmed using flow cytometry as well as confocal microscopy (Fig. 2C-D). Importantly, after 24-hr incubation, a near-complete removal of dead material was observed. This was an active process that did not occur at 4°C.

Indirect allorecognition studies were performed using HLA-A2-/HLA-DR1+ typed moDC. Whereas strong IFN-γ production was achieved with exogenous peptide loading, none of the conditions investigated resulted in detectable activation of the 4.44 clone (Fig. 2E). Activation of moDC using different toll-like receptor (TLR) ligands did not result in detectable indirect presentation (data not shown). Comparable to the fragments, a low albeit significant response was found for semi-direct allorecognition by 1E2 when necrotic SAL-A2 cells were used (Fig. 2F).

HLA Monomers Can Be Used for Antigen Loading Resulting in Indirect Allopresentation

As an alternative source of alloantigens, we investigated the potential use of HLA-A2 monomers. Incubation of HLA-DR1+/HLA-A2- moDC with HLA-A2 led to potent activation of clone 4.44 comparable to exogenously loading of peptides (Fig. 3A).

This response was dose dependent, and significant IFN-γ production was observed with concentrations starting at 1 μg/mL HLA-A2 and kept increasing until 125 μg/mL (Fig. 3B).

MoDCs were incubated with 25 μg/mL HLA-A2 for various time points, and after extensive washing, 4.44 was added for an additional 24 hr. The highest reactivity was observed when moDCs were incubated for at least 4 hr with the antigen (Fig. 3C). Next, moDCs were incubated with

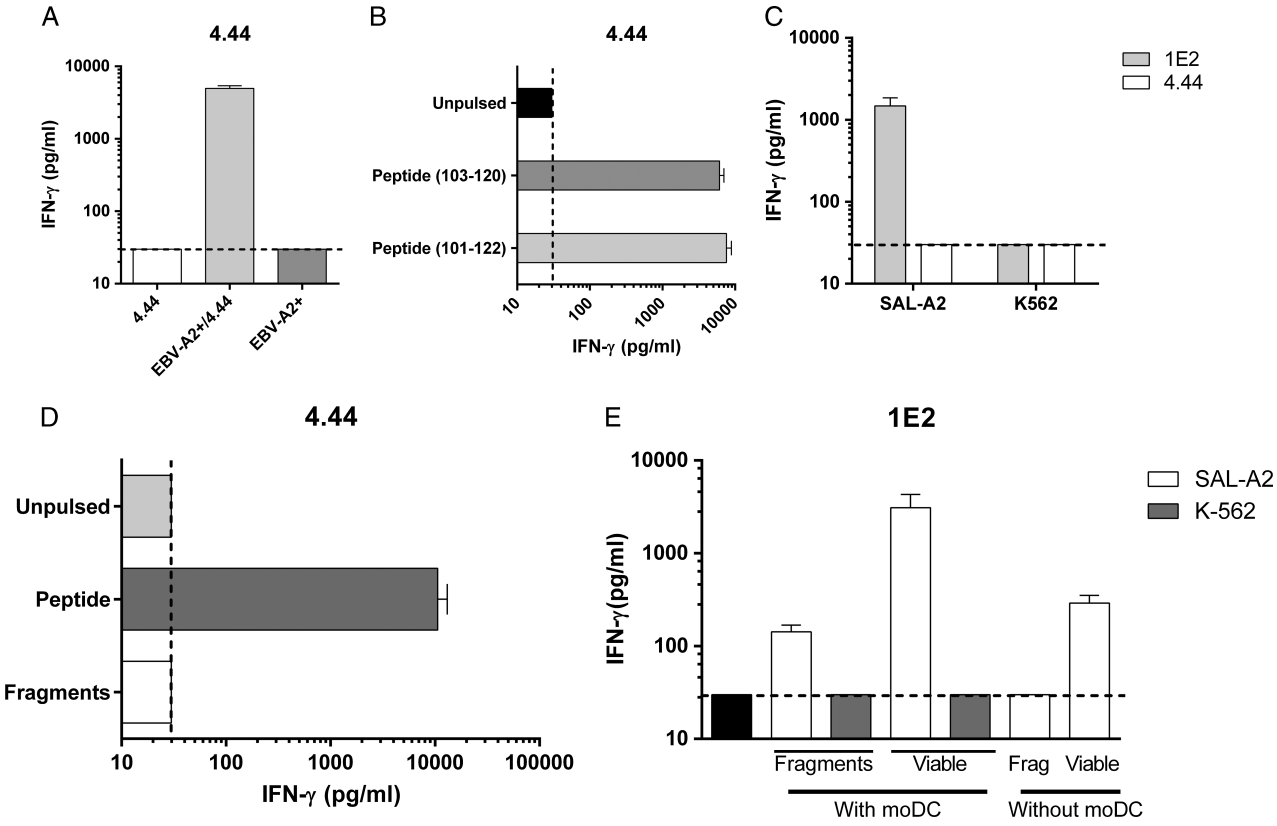

FIGURE 1. T-cell clones recognize cellular fragments through semidirect allorecognition but not through indirect allorecognition. A, 5×10^3 CD4 T-cells (4.44) were cultured with their native ligand HLA-A2+/HLA-DR1+EBV-LCLs. IFN-γ secretion was measured in the supernatants after 24 h. B, HLA-A2-/HLA-DR1+EBV-LCLs were pulsed with different HLA-A2–derived peptides and cocultured with 4.44 T-cells. After 24 hr, IFN-γ was measured in the supernatants. C, An HLA-A2 recognizing CD8 T-cell clone (1E2) and the 4.44 were cocultured with K562 or HLA-A2–transfected K562 cells (SAL-A2) for 48 hr. Supernatants were then collected and IFN-γ measured. D, HLA-typed moDC (HLA-DR1+/HLA-A2-) were cultured with or without fragments of SAL-A2 cells (1:1 ratio at 5×10^4 cells) for 48 hr after which, 5×10^3 4.44 T-cells were added with or without HLA-A2 peptides. E, moDCs were also cultured with 1E2 cells (as described in D). All experiments were repeated three times, graphs represent the mean +SD of a single experiment conducted in triplicate. Dashed lines represent the detection limit of the ELISA used.

HLA-A2 for 4 hr before extensive washing. 4.44 T-cells were then added at 0, 24, or 48 hr and incubated for an additional 24 hr (Fig. 3D). The highest reactivity was observed when 4.44 was added immediately (0 hr), whereas a steady decrease was observed after 24 and 48 hr, indicating a steady turnover of HLA peptide complexes.

Monocytes are the major APCs within the PBMCs; we investigated their capacity to present alloantigens via the indirect pathway. Similar to moDC, monocytes showed a strong capacity to present HLA-A2 and activate 4.44 T-cells, comparable to the exogenous peptide (Fig. 3E–F). This response was dose dependent, and significant levels of IFN-γ were observed at concentrations of 1 μg/mL HLA-A2, reaching an optimum at 25 μg/mL (Fig. 3F).

PBMCs Can Present Monomer-Derived Peptides to Indirect Allorecognizing T-cells Clones Without Evidence for Semidirect Allorecognition

Because the afore mentioned experiments were performed with purified populations, we next addressed the question whether the use of total PBMC would affect the sensitivity or specificity of the above-described model. HLA-DR1+/HLA-A2- PBMCs were pulsed with HLA-A2 peptides or incubated with HLA-A2 monomers in the presence/absence of 4.44. Again using either exogenous peptide or HLA-A2 monomers, a strong and dose-dependent response of the T-cell clone could be detected (Fig. 4A–B). In the absence of the T-cell clone, no IFN-γ production could be detected, indicating that the addition of HLA-A2 monomer does not activate CD8 T-cells with direct allospecificity (Fig. 4A). The absence of semi-direct allorecognition could be further confirmed with the 1E2 clone, which was completely unresponsive when incubated with the HLA-typed PBMCs and increasing concentrations of HLA-A2, as depicted by IFN-γ production as well proliferation (Fig. 4C–D). This is in stark contrast to clone 4.44, which was highly positive in both conditions.

To investigate the sensitivity of this model, various PBMCs concentrations were tested and incubated for 24 or 48 hr with a set amount of HL-A2 and 4.44 (Fig. 4E); 48 hr and 1×10^5 PBMCs yielded overall a better T-cell response. When titrating the T cell clone, as little as 8 to 40 T-cells were sufficient to induce detectable levels of IFN-γ when 1×10^5 PBMCs were incubated with 25 μg/mL HLA-A2 for 48 hr (Fig. 4F).

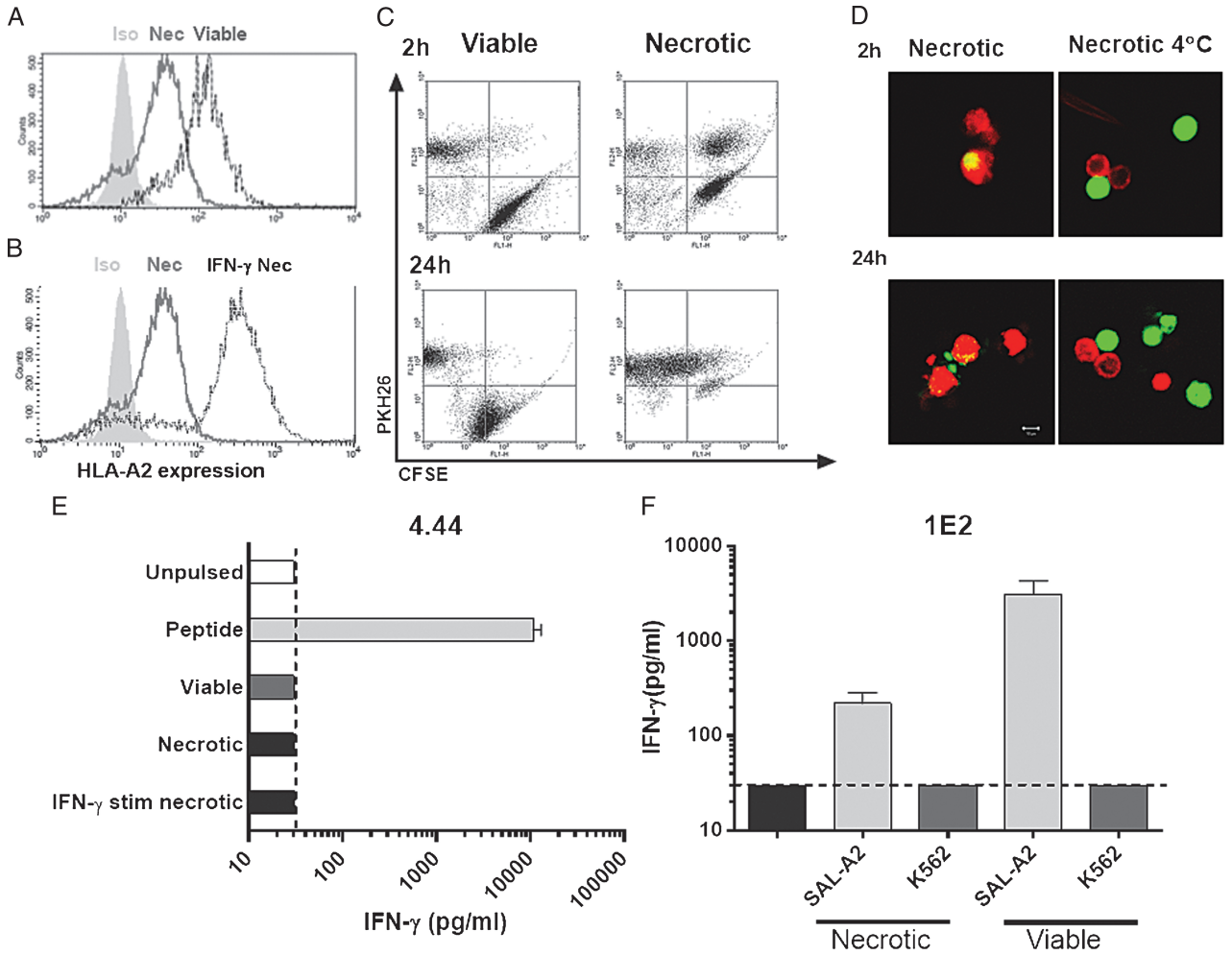

FIGURE 2. T-cell clones recognize necrotic cells through semi-direct allorecognition but not through indirect allorecognition. A–B, SAL-A2 cells were stained with HLA-A2-PE-labeled antibodies or isotype control. Cells were either viable or necrotic. IFN-γ–stimulated SAL-A2 cells were stimulated with 100 ng/ml IFN-γ for 24 hr prior to HLA-A2 labeling. C–D, moDCs were labeled with PKH26 and SAL-A2 cells were labeled with CFSE. Necrosis was then induced, and as a control, viable cells were used. 1×10^4 SAL-A2 cells were incubated in a 1:1 ratio with moDCs for 2 or 24 hr. Uptake of necrotic cells by moDCs was quantified using flow cytometry doubled positive populations in the dotplots represent phagocytosis by moDCs, which was also confirmed using confocal microscopy. E, Typed moDC (HLA-DR1+/HLA-A2-) were cultured with living/necrotic or IFN-γ–stimulated necrotic SAL-A2 cells (1:1 ratio at 5×10^4 cells) for 48 hr, after which, 5×10^3 4.44 T-cells were added with or without HLA-A2 peptides. F, moDCs were also cultured with 1E2 cells (as described in E). All experiments were repeated three times; graphs represent mean + SD of a single experiment conducted in triplicate. Confocal imaging was repeated twice. Dashed bar depicts the detection limit.

Finally, we could confirm that antigen presentation was completely HLA-DR dependent. The cytokine production and proliferation were completely and dose-dependently prevented when cultures with 4.44 were incubated with a blocking antibody against HLA-DR (Fig. 4G–H).

DISCUSSION

Indirect allorecognition is considered one of the important factors in mediating chronic allograft rejection and influencing long-term graft outcome (*7, 41–43*). However, at the moment, no reliable test is available to monitor indirect allorecognition in organ transplant recipients. A major hurdle is the antigen loading that requires a high sensitivity to detect low-frequency indirect T-cells and a high specificity to exclude T-cells with direct specificity. In the current study, we have successfully developed a novel in vitro method to measure indirect allorecognition. By using T-cell clones that specifically recognize HLA-A2 through the indirect (HLA-A2 peptides restricted by HLA-DR1) or direct (HLA-A2) pathway of allorecognition, we could demonstrate that semidirect allorecognition is not an issue in our model system, where HLA-A2 monomers were used as a source of alloantigen.

In experiments using HLA-A2 expressing cells as donor antigen source (either as cell fragments or as necrotic cell), reactivity was limited to the semidirect pathway of

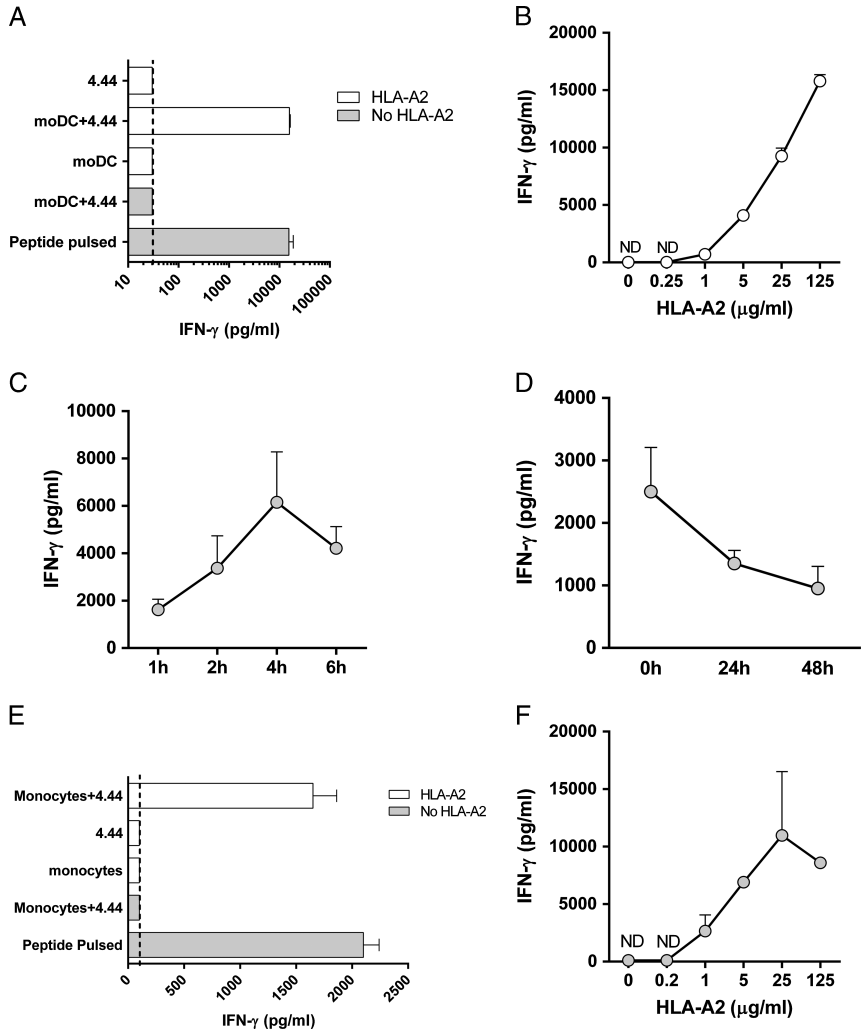

FIGURE 3. moDCs and monocytes present monomers to T-cell clones in a time- and dose-dependent manner. A–B, HLA-DR1+/HLA-A2- moDCs were incubated with HLA-A2–derived peptides(peptide pulsed) or HLA-A2 monomers(HLA-A2) in cocultures with or without the 4.44 T-cells for 24 hr. Supernatants were collected and IFN-γ measured. HLA-A2- moDCs presented HLA-A2 monomer–derived peptides in a dose-dependent manner to the T-cells. C, HLA-typed moDCs were incubated with HLA-A2 monomers for 1,2,4, and 6 hr, then washed thoroughly before the 4.44 T-cells were added for an additional 24-hr incubation. After a 24-hr incubation, IFN-γ was measured in the supernatants. D, HLA-typed moDCs were incubated with HLA-A2 monomers for 4 hr and then thoroughly washed. T-cells were then added immediately (0 hr), 24 hr or after 48 hr. Supernatants were harvested after 24 hr. E–F, HLA-DR1+/HLA-A2- monocytes were pulsed with an HLA-A2–derived peptide or different concentrations of monomers (HLA-A2) and cocultured in the presence or absence of 4.44 T-cells. After 24-h incubation, IFN-γ was measured in the supernatants. All experiments were conducted in triplicate; results indicate the mean+SD of one experiment in triplicate. Dashed bars indicate the detection limit of the ELISA used. ND, not detectable.

alloreactivity. This raises some questions on the conclusions drawn from previous studies that have routinely used cellular fragments to measure indirect alloreactivity (*7, 10, 44*). Those studies did not include controls to distinguish between semidirect and indirect allorecognition, and it is therefore possible that the semidirect pathway has a contribution to the allorecognition measured in those studies.

The semidirect pathway involves the incorporation of alloantigen intact on the cell surface and has been shown to occur with DCs (*45*), monocytes, and T-cells (*46, 47*). The process of membrane transfer is a subject of many studies, and the mechanisms involved are now slowly unraveled (*48*). Interestingly, cross-dressed DCs (DCs that express an acquired HLA-peptide complex) have been shown to prime CD8 T-cells in mice (*45, 49*). This suggests that semidirect allorecognition could potentially lead to T-cell priming, which is an important factor in interpreting model systems that use cell fragments or apoptotic and necrotic cells to monitor indirect allorecognition. Reactivity against HLA class I molecules was observed in three of five experiments performed. The reactivity was limited to low levels of IFN-γ and is most likely due to the limited HLA class I molecules

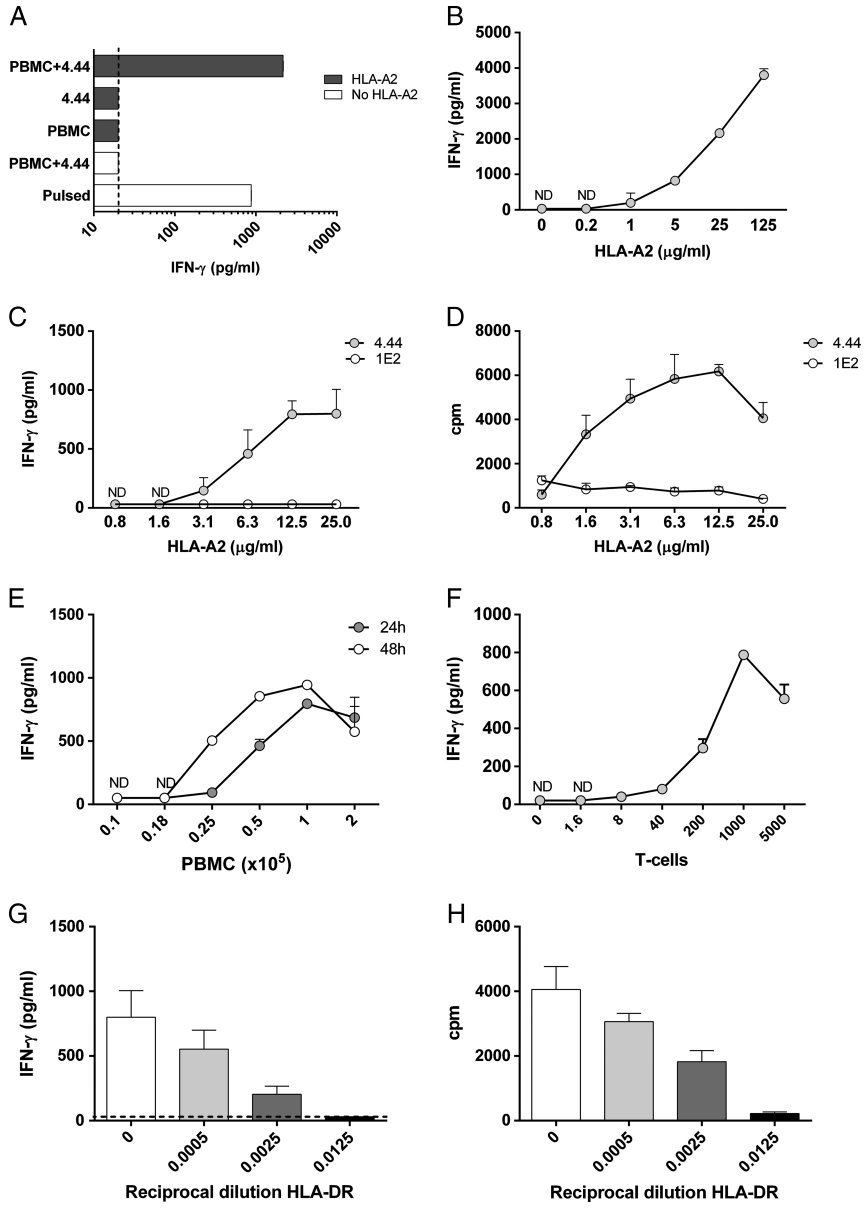

FIGURE 4. PBMCs can present HLA-A2 monomer derived peptides via HLA-DR1 with no measurable semi-direct alloreactivity. A–B, 1×10^5 Typed HLA-DR1+/HLA-A2- PBMCs were incubated with different concentrations of HLA-A2 monomers or pulsed with an HLA-A2 peptide and cocultured with or without 5×10^3 4.44 T-cells; 48 hr after incubation, supernatants were collected and IFN-γ measured. C–D, PBMCs were incubated with different concentrations of HLA-A2 monomers and cocultured with either the indirect recognizing clone 4.44 (gray circles) or with the direct recognizing clone 1E2 (white circles) for 48 hr. IFN-γ and proliferation were analyzed as previously described. E, Different PBMC numbers were incubated with 25 μg/mL HLA-A2 in the presence of 5×10^3 4.44 for 24 or 48 hr. Supernatants were then harvested and IFN-γ measured. F, To test the minimal amount of T-cells needed to detect a response, 4.44 were titrated, and 25 μg/mL HLA-A2 monomer was added in combination with 1×10^5 HLA-typed PBMCs for 48 hr. Supernatants were then collected and IFN-γ measured. G–H, PBMCs were cocultured with 4.44 T-cells in the presence of 25 μg/mL HLA-A2 for 24 hr with or without anti-HLA-DR monoclonal antibodies (mAb). The reciprocal dilution of the HLA-DR mAb is set on the x-axis. All experiments were conducted at least three times. Shown is the mean+SD of one experiment in triplicate. Dashed line represents the detection limit of the ELISA used. ND, not detectable.

transferred. The conditions in most experiments involving membrane transfer are short term (2 hr); it is possible that longer periods (as used in our experiments) reduce the efficiency and thereby the reactivity. However, we observed no indirect alloreactivity in any of the conditions using cellular fragments or dead cells. Although the material is taken up and processed, there is relatively little HLA class I molecules when compared with all other proteins available that will

also be processed and presented and therefore will generate large amounts of competing peptides that will not be recognized by the T cell clone.

Another strategy to monitor indirect presentation has been the use of synthetic peptides. In vivo APCs present donor-derived peptides to autologous T-cells restricted by their own HLA class II molecules. Each HLA class II molecule has its own repertoire of peptides that can be presented. Prediction of HLA class II epitopes is complex, and it is unlikely that a set of peptides can be used to predict individual alloreactivity (*50*). Current tests that use synthetic peptides have the risk of creating neo-epitopes, epitopes that are not created in the natural antigen processing manner (*31–33, 51*). This may even lead to reactivity to peptides derived from autologous HLA molecules. Peptides used for the detection of indirect allorecognition include peptides derived from the β1 domain of HLA class II (*32, 51–53*) or the α1 domain of HLA class I (*33, 54*). In one study, where peptides from a complete HLA-A2 molecule were used, reactivity was seen in the hypervariable region as well as from the α3 subunit and the transmembrane domain (*52*). However, as no peptides from self-HLA were taken as a control, it is unclear what the reactivity means and whether these peptides would be formed when naturally processed. Use of longer peptides could be a good alternative, as these would require processing and are able to induce T-cell reactivity in other model systems of antigen presentation (*53, 54*).

HLA monomers offer solutions to many of the problems depicted earlier. However, there are two distinct disadvantages in using monomers. The one is the high price involved in making highly pure monomers and, second, the relatively high concentration needed to induce a reproducible T-cell response.

The quest to induce long-term graft tolerance and/or survival starts with an accurate depiction of the recipient's immune response against the donor allograft. Although many studies are aimed at monitoring and measuring operational tolerance and organ rejection (*55, 56*), no reliable test exists other than the actual clinical outcome. Measuring indirect allorecognition could give a broad view into how the recipient immune system is shaping its response to the allograft and be helpful in tailoring an individual immune-suppression regime to prolong graft survival.

MATERIALS AND METHODS

Cell Culture and Reagents

Peripheral blood mononuclear cells (PBMCs), monocytes, and monocytes-derived dendritic cells (moDCs) were cultured in RPMI-1640 (PAA, Austria) supplemented with 10% FCS (Bodinco, The Netherlands), 5,000 U/mL penicillin, and 5 mg/mL streptomycin (Invitrogen, USA).

Single antigen lines (SAL) were created by transfecting K562 cells with a plasmid construct containing a HLA-A2 heavy chain gene (SAL-A2) (*57*). SAL-A2 were maintained in IMDM supplemented with 10% FCS, pen/strep, and G-418 (Roche, Germany) at a concentration of 250 μg/mL for the selection of stably transfected SALs.

HLA-A*0201–derived peptides of the region 99-122 of HLA-A2 were synthesized. HLA-A*0201 heavy chains were produced in *Escherichia coli*. Monomer refolding around the melanoma-associated pmel 17 peptide (YLEPGVTA) in the presence of β2-microglobulin was achieved as previously described (*58*). Monomers were purified using gel filtration HPLC and tested routinely.

T-cell clones 4.44 (CD4+) and 1E2 (CD8+), recognizing HLA-A2 (aa98–120) restricted by HLA-DR1 and HLA-A2, respectively, have been previously described (*38, 59*). They were maintained in IMDM medium (PAA, Austria) with 5% FCS (Bodinco, The Netherlands), 5% normal human serum (Sanquin, The Netherlands), 100 IU/mL recombinant IL-2 (Chiron, Novartis, USA) 5,000 U/mL penicillin, 5 mg/mL streptomycin, and 2 mM L-glutamine (Gibco, Invitrogen, USA). Expansion was achieved by stimulation with phytohemagglutinin (PHA, 0.8 μg/mL, Murex Biotec Limited, Dartford, UK) and PBMCs in a ratio of 1:5. Cells were harvested after 2 weeks and either frozen or used in experiments after a resting period of 2 to 3 days. T-cell specificity was routinely tested.

Stable EBV-transformed B-cell lines (EBV-LCL) were generated from an HLA-DR1+/HLA-A2- donor using standard procedures (*60*). HLA-A2+ EBV-LCLs were generated by transducing a retroviral vector encoding for HLA-A*0201 into the donor (HLA-A2-) EBV-LCLs (*61*).

Generation of Monocyte-Derived Dendritic Cells

moDCs were generated from buffy coats as previously described (*62*). Briefly, PBMCs were isolated from buffy coats (Sanquin, The Netherlands) of healthy ($HLA\text{-}A2^-$,$HLA\text{-}DR1^+$) individuals using Ficoll/amidotrizoaat (pharmacy, LUMC, The Netherlands) density gradient, followed by CD14 microbeads magnetic cell sorting (Miltenyi Biotec, The Netherlands) according to the manufacturer's protocol. Monocytes were cultured in six-well plates (Costar, USA) in RPMI-1640 supplemented with 10 ng/mL IL-4 and 5 ng/mL GM-CSF (Gibco, Invitrogen, USA). Cytokines were refreshed every 2 to 3 days, and cells were allowed to differentiate for at least 6 days before harvesting.

Indirect Allorecognition Assay Using Cells as Source of HLA Class I Antigens

moDCs were cocultured with necrotic, apoptotic, or fragmented SAL-A2 cells. Necrosis and apoptosis was induced as previously described (*40*). Briefly, necrosis was induced by heating cells to 56°C for 1 hr and confirmed using light microscopy and annexin-V/PI staining. Cell fragments were generated using three rounds of freeze-thawing and confirmed using light microscopy; 5×10^5 moDCs were cocultured at a 1:1 ratio with SAL-A2 cells for a period of at least 24 hr in a 96-round well plate, and 5×10^3 4.44 (CD4 indirect) or 1E2 (CD8 direct/semidirect) cells were added for an additional 48-hr incubation. Supernatants were then harvested, and IFN-γ production was measured.

Phagocytosis Assay

Phagocytosis was quantified using flow cytometry or fluorescence microscopy as previously described (*40*). Briefly, moDCs were labeled with PKH26 (Sigma-Aldrich) or stained with HLA-DR mAb. Necrotic, apoptotic, or fragmented SAL-A2 cells were stained with CFSE before induction of cell death and then cocultured with moDCs at a ratio of 1:1 (5×10^4 cells). Analysis was conducted at 2 or 24 hr post coculture. Fluorescence was assessed with FACSCalibur or LSR-II or with a Leica SP5 confocal scanning laser microscope, and the analysis preformed with ImageJ imaging software.

Indirect/Direct Allorecognition Assay Using HLA Class I Monomers

To monitor the pathways of allorecognition moDC or monocytes ($DR1^+/A2^-$) were plated (3×10^4) in round 96-well plates (Costar, USA) and incubated with different concentrations of HLA-A2 monomers (HLA-A2) or peptides. T-cells were added to the culture and incubated at different time points. A CD4 T-cell clone (5×10^3, 4.44) was used as a readout for indirect allorecognition and a CD8 T-cell clone (5×10^3, 1E2) for direct/semidirect allorecognition. Part of the supernatant was harvested, and cells were pulsed with 0.5 μCi ^3H (Life Science Products, Inc., USA) and incubated for 24 hr. Similarly, (1×10^5) PBMCs were cocultured with (5×10^3) T-cells (4.44 or 1E2) with different concentrations of HLA-A2. Cocultures were incubated for 48 hr, supernatants collected, and cells pulsed with ^3H for another 24 h when proliferation was measured. Inhibition of indirect allorecognition was achieved by titration of mouse antihuman HLA-DR (B8.11.2, IgG2b) antibodies to the cocultures.

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